

$\alpha_1\beta_1$ Integrin on Neural Crest Cells Recognizes Some Laminin Substrata in a Ca^{2+} -independent Manner

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Abstract. Neural crest cells migrate along pathways containing laminin and other extracellular matrix molecules. In the present study, we functionally and biochemically identify an $\alpha_1\beta_1$ integrin heterodimer which bears the HNK-1 epitope on neural crest cells. Using a quantitative cell adhesion assay, we find that this heterodimer mediates attachment to laminin substrata prepared in the presence of Ca^{2+} . Interestingly, neural crest cells bind to laminin- Ca^{2+} substrata in the presence or absence of divalent cations in the cell attachment medium. In contrast, the attachment of neural crest cells to laminin substrata prepared in the presence of EDTA, heparin, Mg^{2+} , or Mn^{2+} requires divalent cations. Interactions with these laminin substrata are mediated by a different integrin heterodimer, since antibodies against β_1 but not α_1 integrins inhibit neural

crest cell attachment. Thus, the type of laminin substratum appears to dictate the choice of laminin receptor used by neural crest cells. The laminin conformation is determined by the ratio of laminin to Ca^{2+} , though incorporation of heparin during substratum polymerization alters the conformation even in the presence of Ca^{2+} . Once polymerized, the substratum appears stable, not being altered by soaking in either EDTA or divalent cations. Our findings demonstrate: (a) that the $\alpha_1\beta_1$ integrin can bind to some forms of laminin in the absence of soluble divalent cations; (b) that substratum preparation conditions alter the conformation of laminin such that plating laminin in the presence of Ca^{2+} and/or heparin modulates its configuration; and (c) that neural crest cells utilize different integrins to recognize different laminin conformations.

THE neural crest is a transient population of cells that are unique to developing vertebrates. These cells arise from the dorsal aspect of the neural tube and migrate through regions rich in extracellular matrix (ECM)¹ molecules. The pathways of neural crest cell migration have been studied extensively in chick embryos using a variety of cell marking techniques (LeDouarin, 1973; Rickmann et al., 1985; Bronner-Fraser, 1986; Serbedzja et al., 1989). After their migration, these cells differentiate into a diverse group of derivatives including melanocytes, neurons and glia of the peripheral nervous system, chromaffin cells, and the facial skeleton.

The environment through which neural crest cells move is thought to influence both their migration and differentiation. Perturbation experiments performed in avian embryos suggest that integrins and a variety of ECM ligands are required for the proper migration of cranial neural crest cells in situ (for review see Lallier and Bronner-Fraser, 1990). Cranial neural crest cell migration is inhibited by microinjection into the head region of antibodies against the β_1 subunit of integrin (Bronner-Fraser, 1985, 1986b), fibronectin (Poole and Thiery, 1986), a laminin-heparan sulfate proteoglycan

(HSPG) complex (Bronner-Fraser and Lallier, 1988), tenascin (Bronner-Fraser, 1988), and the HNK-1 epitope (Bronner-Fraser, 1987). In the axolotl, there is evidence that the ECM can dictate the timing of initial neural crest cell emigration. Microcarriers "conditioned" with ECM by implantation into embryos during advanced stages of neural crest migration can induce precocious emigration of neural crest cells when transplanted into younger embryos (Löfberg et al., 1985). In the "white" axolotl mutant, presumptive pigment cells fail to migrate along a subectodermal pathway, apparently due to a defect in the ECM (Löfberg et al., 1989). Thus, ECM components are important substrata for neural crest cells, and can influence both the timing and the pattern of neural crest migration.

The ECM through which neural crest cells migrate is complex, containing fibronectin (Newgreen and Thiery, 1980; Duband and Thiery, 1982), tenascin/cytotactin (Tan et al., 1987), collagens (Duband and Thiery, 1987; Perris et al., 1991b), proteoglycans (Bronner-Fraser and Lallier, 1988; Perris et al., 1991a), and laminin (Krotoski et al., 1986; Duband and Thiery, 1987). Parallel studies conducted in tissue culture demonstrate that many of these substrata support neural crest cell migration. For example, isolated neural crest cells are motile on both laminin and fibronectin in vitro (Rovasio et al., 1983; Newgreen, 1984; Goodman and Newgreen, 1985; Perris et al., 1989). Interactions between neu-

1. *Abbreviations used in this paper:* ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan; LN, laminin; PVC, polyvinyl chloride.

ral crest cells and laminin appear to be mediated by integrin receptors (Lallier and Bronner-Fraser, 1991) as well as galactosyl transferase (Runyan et al., 1986, 1988).

Integrins are transmembrane, heterodimeric receptors that mediate attachment of cells to a variety of ECM molecules including fibronectin, laminin, vitronectin, various collagens, and tenascin (Horwitz et al., 1985; Buck et al., 1986; Hynes, 1987; Tomaselli et al., 1988; Bourdon and Ruoslahti, 1989). Recently, a number of laminin-binding integrin heterodimers have been identified and isolated in mammalian cells, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ (Forsberg et al., 1990; Hall et al., 1990; Clyman et al., 1990; Kramer et al., 1990; Toyota et al., 1990; Languino et al., 1989; Elices and Hemler, 1989; Carter et al., 1990; Lotz et al., 1990; Kirchofer et al., 1990; Gehlsen et al., 1989; Sonnenberg et al., 1988, 1990; Shaw et al., 1990; Dedhar and Sanlimien, 1990; Shimizu et al., 1990). All integrins are reported to require divalent cations for binding to their ligands, although their individual requirements for divalent cations differ. For attachment to laminin, for example, the $\alpha_3\beta_1$ integrin requires Ca^{2+} , while the $\alpha_6\beta_1$ integrin requires either Mg^{2+} or Mn^{2+} , but not Ca^{2+} (Sonnenberg et al., 1988). Therefore, defining the cation requirements for cell adhesion may be a useful approach for classifying integrins in systems where subunit-specific antibodies are not available. In addition to interacting with cell-surface receptors, laminin binds to a number of ECM components including collagen, nidogen/entactin, HSPG (for review see Timpl et al., 1990), and itself (Yurchenco et al., 1985, 1990).

We have previously shown that neural crest cells can interact with laminin by at least two distinct adhesion mechanisms (Lallier and Bronner-Fraser, 1991). One utilized a divalent cation-independent receptor that was inhibited by the HNK-1 antibody. In the present study, we identify this as an $\alpha_1\beta_1$ integrin heterodimer. The other receptor system utilized a divalent cation-dependent integrin that was insensitive to the HNK-1 antibody and required Ca^{2+} or Mn^{2+} , but not Mg^{2+} , for binding. Our results suggest that neural crest cells use these different integrins to interact with two functionally distinct conformations of laminin. These different laminin substrata are determined by the ratio of laminin to Ca^{2+} and can be modulated by addition of heparin in the plating buffer, suggesting that such substrata could occur under physiological conditions.

Materials and Methods

Materials

Laminin was purified from Engelbreth-Holm-Swarm tumors as described by others (Timpl et al., 1990). Heparin, dextran sulfate, chondroitin sulfate, and ovalbumin were purchased from Sigma Chemical Co. (St. Louis, MO). JG22 hybridoma cells were purchased from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). The HNK-1 antibody-producing hybridoma cell line was purchased from ATCC (Rockville, MD). The anti- α_1 integrin antiserum was the generous gift of Dr. Mats Paulsson.

Neural Crest Cell Primary Cultures

Primary neural crest cultures were prepared from the neural tubes of Japanese quail embryos (*Coturnix coturnix japonica*) as previously described (Lallier and Bronner-Fraser, 1991). Neural crest cells for use in quantitative attachment assays were labeled by the addition of [^3H]leucine (50–100 $\mu\text{Ci}/\text{ml}$) to their culture media 1 h after explantation. The cells were allowed

to incorporate labeled leucine for 12–16 h before use in the assay. The neural crest cells were rinsed five times with blocking MEM (bMEM; 0.5 mg/ml ovalbumin (Sigma Chemical Co., St. Louis, MO) in MEM (Gibco Laboratories, Grand Island, NY)). Cells were removed by incubation in 5 mM EDTA in bMEM for 10–15 min at 37°C.

Substratum Preparation

Laminin was coated at a concentration of 20 $\mu\text{g}/\text{ml}$ in carbonate buffer (100 mM, pH 8.0) onto polyvinyl chloride (PVC) microtiter plates by adsorption for 12–24 h at 25°C. This buffer also contained either 5 mM CaCl_2 , 5 mM EDTA, or 5 mM CaCl_2 with 1 mg/ml heparin as noted for each experiment. Substrata were washed five times with PBS followed by a 2-h incubation with bMEM at 25°C. Substrata were pretreated with 5 mg/ml ovalbumin (Sigma Chemical Co.) to block nonspecific cell attachment.

Cell Attachment Assay

Cell-substratum adhesion was measured using the method first described by McClay et al. (1981), modified for use with avian neural crest cells (Lallier and Bronner-Fraser, 1991). PVC microtiter wells, coated with the appropriate substratum, were rinsed and filled with either bMEM containing the antibody to be tested or blocking phosphate buffer (bPBS; 10 mM phosphate buffer, pH 7.4, with 150 mM NaCl and 0.5 mg/ml ovalbumin) containing the divalent cation or EDTA concentration to be tested. Neural crest cells (between 200 and 500 per well) were brought into contact with the substratum by a centrifugal force of 150 g for 5 min. Chambers were sealed and incubated for 15 min at 37°C or 4°C. Unbound neural crest cells were removed by a centrifugal force of 50 g for 5 min. The chambers were quick frozen in a methanol/dry ice bath. The uncoated portion (unbound cells) and substratum-coated portion (bound cells) of each well were removed and placed separately into scintillation vials. Samples were analyzed for ^3H cpm using a scintillation counter (LS 5801; Beckman Instruments, Inc., Fullerton, CA). Individual wells contained between 5,000 and 30,000 cpm with background counts on the order of 20–50 cpm. The percentage of adherent neural crest cells per well was determined as follows:

$$\% \text{ Cells bound} = \% \text{ cpm} = \frac{\text{cpm bound to substratum (bottom)}}{\text{Total cpm (bottom + top)}} \times 100\%$$

For the experiments described, nonspecific binding to ovalbumin ranged from 5–15% of the counts present within a given well. In studies looking at antibody inhibition of cell attachment, the data have been simplified to express the percent inhibition of attachment resulting from exposure to the antibodies.

$$\% \text{ Inhibition} = \frac{(\% \text{ Cells bound without antibodies}) - (\% \text{ Cells bound with antibodies})}{(\% \text{ Cells bound with antibodies})}$$

Data points represent the SEM for six wells within one experiment, and each experiment was repeated at least three times. Variability between experiments using identical conditions was $\sim 10\%$. Values for percentage of neural crest cells bound were deemed significantly different when the p value, determined by Student's one-sided t test, was <0.05 .

Substratum Preference Assay

Lanes of laminin substrata were prepared in polystyrene dishes as described by Walter et al. (1987). 35-mm tissue culture dishes (Falcon) were precoated with 100 μl of a nitrocellulose solution (2.5 cm^2 nitrocellulose in 10 ml methanol), and allowed to air dry. Alternating stripes of laminin substrata were coated on these dishes by placing a silicon mold onto the surface of the dish which allowed the primary substrata to come into contact only with the plastic in 50- μm stripes. The primary substrata was mixed with 10 $\mu\text{g}/\text{ml}$ Texas Red stain coupled to BSA (Sigma Chemical Co.), to allow later visualization of the different substrata on an inverted fluorescence microscope. Primary substrata were coated with three changes of protein-coating solution, which was incubated for 20 min per coating. Primary substrata were blocked with 1 mg/ml ovalbumin in 50 mM carbonate buffer for 20 min. Secondary substrata were added to dishes after the removal of the silicon mold, and allowed to coat the plastic for 1 h. Substrata were again blocked with 1 mg/ml ovalbumin for 1 h. Suspensions of neural crest cells were allowed to settle onto both substrata by incubating a 100 μl drop of MEM containing 10^3 cells with the substrata for 2 h. At this point, the polystyrene dishes were filled with MEM containing the indicated concentrations of EDTA, and incubated for 12 h. Cells were fixed with 4% parafor-

maldehyde and viewed using a Diaphot inverted fluorescence microscope (Nikon Inc., Melville, NY).

Measurement of Laminin Bound to PVC Plates

Radiolabeling of laminin was performed by reductive methylation as previously described by Tack et al. (1980). Briefly, a protein solution at a final concentration between 1 and 5 mg/ml was dialyzed against 200 mM borate buffer, pH 8.9, and 500 μ l was placed in glass vials on ice for 1 h. 500 μ l of 180 mM formaldehyde was added to the sample, followed by 100 μ l [3 H]NaBH₄ (2 mCi in 0.1 N NaOH), and allowed to react for 1 h on ice. The unbound [3 H]NaBH₄ was removed by passing the protein sample through a disposable Sephadex G-25M column (Pharmacia Fine Chemicals, Piscataway, NJ). Protein concentration was assayed using the Bio-Rad protein assay following the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA). Labeled proteins were serially diluted in carbonate buffer, pH 8.0, containing either 5 mM Ca²⁺ or 5 mM EDTA, and applied to wells in PVC microassay plates, where they were allowed to bind for 12 h at 25°C. Wells were washed five times with carbonate buffer, separated, and placed into scintillation vials for analysis.

Immunoprecipitation of α_1 Integrin and HNK-1 Antigen from Surface-labeled Neural Crest Cells

Immunoprecipitations were performed on cultures of surface-biotinylated neural crest cells as detailed by Lallier and Bronner-Fraser (1991). Proteins were extracted from these cells using an extraction buffer (20 mM Tris, pH 7.2, 150 mM NaCl, 1 mM CaCl₂, 1% Triton X-100, 1 mM PMSF) for 1 h at 4°C. Extracts were cleared twice by incubation with Sepharose 4B for 1 h at 4°C. Antibodies were added to the extracts and incubated for 12 h at 4°C, followed by incubation with protein A-Sepharose for 1 h at 4°C. Antibody-antigen protein A complexes were washed extensively with wash buffer (20 mM Tris, pH 7.2, 150 mM NaCl, 1 mM CaCl₂, 0.1% Triton X-100, 1 mM PMSF). HNK-1 immunoprecipitations were performed using purified antibody covalently coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals) prepared using the manufacturer's instructions.

Immunoprecipitates were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose (Burnette, 1981). Apparent relative molecular masses were estimated by comparison to prestained high-range molecular weight standards (Gibco-BRL, Bethesda, MD). Membranes were blocked with 5% BSA before incubation with ¹²⁵I-streptavidin (Amersham International, Amersham, UK) for 1 h in TBS/Tween (10 mM Tris, pH 7.2, 150 mM NaCl, and 1% Tween 20) containing 1% BSA. Membranes were air dried, and exposed to X-ray film (XAR5, Kodak) at -70°C, which was developed using D19 developer (Eastman Kodak Co., Rochester, NY). Immunoprecipitates of neural crest cells with the HNK-1 and anti- β_1 integrin (JG22) antibodies were probed with the anti- α_1 integrin antiserum in immunoblots as described previously (Lallier and Bronner-Fraser, 1991). Anti- α_1 integrin antibodies were visualized using ¹²⁵I-protein A.

Results

Previously, we observed that neural crest cells interacted with laminin using different adhesion mechanisms depending upon the concentration of laminin in the solution used to create the substratum (Lallier and Bronner-Fraser, 1991). A possible explanation for this observation was that neural crest cells were differentially recognizing laminin deposited in different configurations. Here, we tested this possibility by characterizing the properties of cell attachment to the prepared laminin substrata using different coating conditions. In addition, we explored the nature of the integrins that mediate neural crest cell attachment to these different laminin substrata. To analyze neural crest cell interactions with laminin, we used a centrifugal cell attachment assay (McClay et al., 1981; Lallier and Bronner-Fraser, 1991) which allows accurate and reproducible measurements of cell-matrix interactions with the relatively small number of cells obtained from primary neural crest cultures.

Laminin Coating Conditions

Laminin substrata were prepared in the presence of (a) 5 mM Ca²⁺ (LN-Ca²⁺), (b) 5 mM EDTA (LN-EDTA), or (c) 100 μ g/ml heparin plus 5 mM Ca²⁺ (LN-Heparin). Because these different plating solutions may alter the binding of laminin to plastic, it was important to quantitate the amount of laminin bound to the PVC plates under different conditions. Radioactively labeled laminin (0.2–100 μ g/ml) was added to the PVC plates in the coating solutions listed above. In all three buffers, the same amount of laminin bound to the PVC wells for a given laminin concentration (data not shown). Thus, the plating conditions did not alter laminin's ability to bind to plastic. A laminin concentration of 20 μ g/ml was chosen for all subsequent experiments.

Initial Neural Crest Cell Adhesion and Stable Cell Attachment in the Presence of Divalent Cations

Initial adhesion, the initial interaction between the cells and the substratum, can be measured by performing the centrifugal binding assay at 4°C. This temperature inhibits receptor lateral mobility, receptor insertion, and microtubule formation. Initial adhesion contrasts with stable attachment of cells to the substratum, which will subsequently be referred to as "attachment." The latter results in strengthened cell-substratum contact mediated by a complex series of events including cytoskeletal reorganization and formation of focal contacts. Attachment was measured on cells incubated for 15 min at 37°C, which permits stabilization and strengthening of attachment to occur.

Initial adhesion was compared to attachment of neural crest cells on the three laminin substrata: LN-EDTA, LN-Ca²⁺, and LN-Heparin. Neural crest cells were added to these substrata in MEM containing 1 mM Ca²⁺ and 500 μ M Mg²⁺. To facilitate rapid comparison of results, we will define "avidity" of binding as the gravitational force required to remove 50% of the cells (A_{50}). For both stable attachment and initial adhesion, all three laminin substrata displayed comparable avidities (Fig. 1), suggesting that neural crest cells interact equally well with all three laminin substrata. However, the avidity of attachment (A_{50} = 700 g) was greater than that for initial adhesion (A_{50} between 10 and 20 g). Thus, at physiological temperatures there was a dramatic strengthening of cell attachment within a few minutes (15 min) of initial adhesion.

Effects of Antibodies and Exogenous Heparin on Neural Crest Cell Interactions in the Presence of Divalent Cations

JG22 Antibody. The JG22 antibody recognizes and inhibits the function of the β_1 subunit of avian integrins. Neural crest cell initial adhesion (Fig. 2 A) and attachment (Fig. 2 B) to LN-EDTA, LN-Ca²⁺, and LN-Heparin substrata were completely abolished by JG22 antibody concentrations of 50 μ g/ml. These results indicate that integrins are involved in the formation of initial contacts and stable attachments of neural crest cells to all three laminin substrata. Furthermore, β_1 integrins may be their sole receptors for attachment to the laminin substrata.

HNK-1 Antibody. In contrast to the nonselective inhibition of neural crest cell attachment and initial adhesion (Fig. 2) by the JG22 antibody, the three laminin substrata exhib-

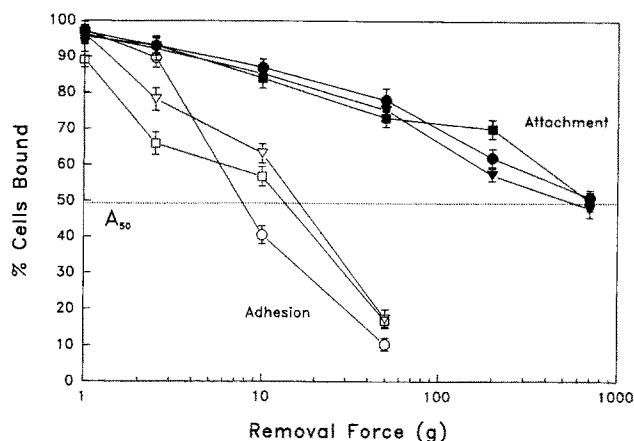


Figure 1. Comparison of neural crest cell attachment (37°C; closed symbols) and initial adhesion (4°C; open symbols) to various laminin substrata; all experiments were performed in the presence of soluble divalent cations. The relative strengths of cell attachment and adhesion were measured by varying the removal force exerted on the cells adhering to laminin plated with Ca^{2+} (LN- Ca^{2+} , triangles), EDTA (LN-EDTA, circles), or heparin plus Ca^{2+} (LN-Heparin, squares). Points represent the mean of at least six wells and the error bars represent the SEM.

ited different sensitivities to the HNK-1 antibody. Both initial adhesion and attachment to LN- Ca^{2+} were inhibited partially (~50%) by the HNK-1 antibody, while attachment to LN-EDTA and LN-Heparin were not significantly affected (concentrations of 50 $\mu\text{g}/\text{ml}$; $p > 0.05$; Fig. 2). Thus, neural crest cell binding to LN- Ca^{2+} is HNK-1 sensitive, while binding to LN-EDTA or LN-Heparin is not. Previously, we have shown that the HNK-1 antibody identifies a $165 \times 10^3 M_r$ integrin or integrin-associated molecule on neural crest cells (Lallier and Bronner-Fraser, 1991).

Anti- α_1 Antiserum. An antiserum that recognizes the $165 \times 10^3 M_r$ α_1 subunit of integrin from chicken gizzard (Syfrig et al., 1991) partially inhibited initial adhesion (Fig. 2A) and attachment (Fig. 2B) to LN- Ca^{2+} , but not LN-EDTA, or LN-Heparin. This inhibition was comparable to that observed with the HNK-1 antibody ($p > 0.05$). When the HNK-1 and anti- α_1 antibodies were added in combination, no significant increase in inhibition of either adhesion or attachment was observed ($p > 0.05$).

Exogenous Heparin. HSPGs have been shown to modulate the attachment of many cell types to laminin (Charonis et al., 1988). Exogenous heparin (1 mg/ml) was added to the media in the assay wells as a competitive inhibitor of cell-surface HSPGs. Attachment and initial adhesion to LN-EDTA, LN- Ca^{2+} , or LN-Heparin were unaltered by exogenous heparin ($p > 0.05$; Fig. 2).

Neural Crest Cell Attachment to LN- Ca^{2+} Occurs in the Absence of Divalent Cations

Previously, we showed that neural crest cells possess at least two distinct attachment mechanisms for laminin: one mediated by a Ca^{2+} -dependent integrin and the other mediated by a divalent cation-independent integrin (Lallier and Bronner-Fraser, 1991). Here, we tested whether neural crest cells could interact with the various laminin substrata in the ab-

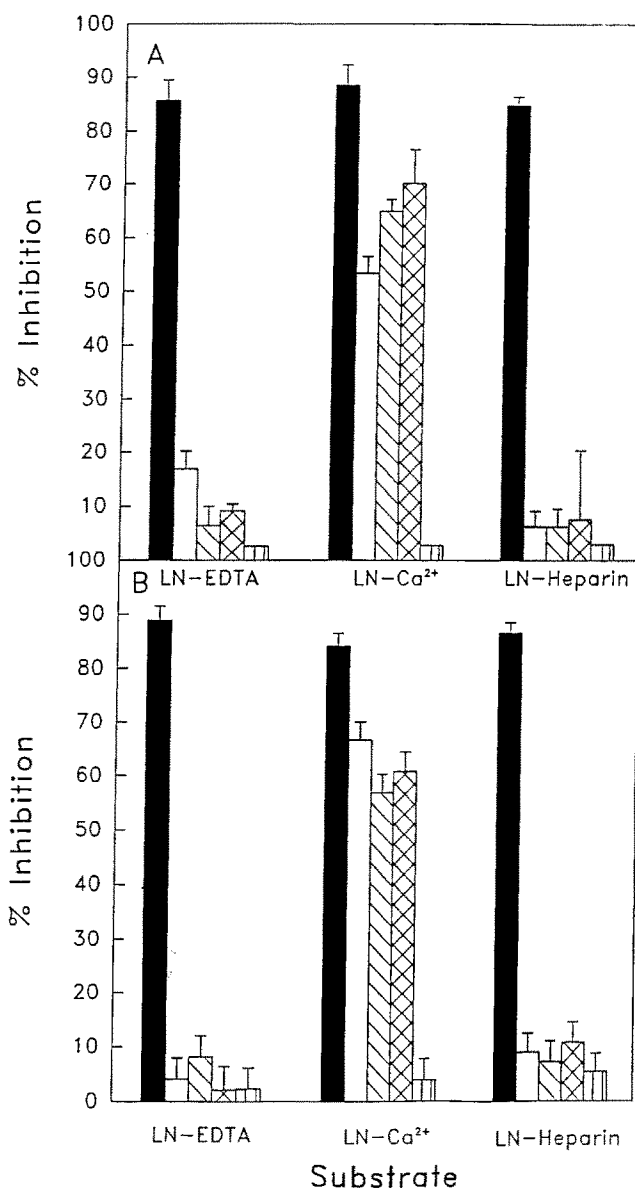


Figure 2. Effects of antibodies and heparin on neural crest cell attachment (A) and initial adhesion (B) to three laminin substrata (LN- Ca^{2+} , LN- Ca^{2+} EDTA, and LN-Heparin); all experiments were performed in the presence of soluble divalent cations. JG22 and HNK-1 antibodies were added to cells at 50 $\mu\text{g}/\text{ml}$. Anti- α_1 antiserum was added to the cells at a dilution of 1:100. Heparin was added at a concentration of 1 mg/ml. The removal force exerted on the cells was 50 g. Bars represent the mean of at least six wells and the error bars represent the SEM. (■) JG22; (□) HNK-1; (▨) anti- α_1 ; (▩) anti- α_1 + HNK-1; (▧) heparin.

sence of divalent cations. Neural crest cells were added to the substratum-coated wells in media containing EDTA, Ca^{2+} , Mg^{2+} , or Mn^{2+} (Fig. 3). Attachment in the presence of Ca^{2+} was comparable for all substrata (~65%; $p > 0.05$). However, when plated in medium that lacked divalent cations, neural crest cells attached only to LN- Ca^{2+} . This significant attachment of neural crest cells (~45% cells bound; $p < 0.05$) to LN- Ca^{2+} in the absence of divalent cations was not abolished by soaking the substratum in 10 mM EDTA for 48 h (data not shown) before assay. Thus, the cat-

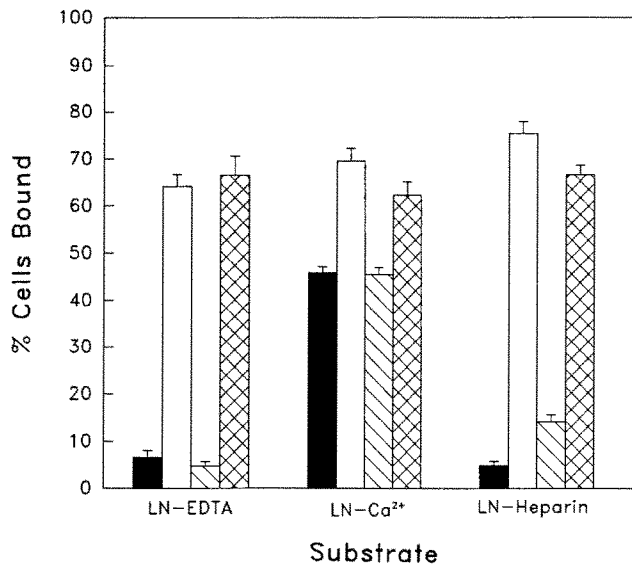


Figure 3. Effects of altering the divalent cations in the medium on neural crest cell attachment to laminin substrata (LN-Ca²⁺, LN-EDTA, and LN-Heparin). The divalent cations Ca²⁺, Mg²⁺, and Mn²⁺ were added to the cell attachment buffer at a concentration of 1 mM, while EDTA was tested at a concentration of 1.5 mM. The removal force exerted on the cells in this experiment was 50 g. Bars represent the mean of at least six wells and the error bars represent the SEM. (■) EDTA; (□) Ca²⁺; (▨) Mg²⁺; (▩) Mn²⁺.

ion independence is unlikely to result from Ca²⁺ leaching off of the LN-Ca²⁺ substratum. Neural crest cell attachment to LN-EDTA and LN-Heparin was similar, both requiring the divalent cations Ca²⁺ or Mn²⁺, but not Mg²⁺. The cation-dependent nature of cell attachment to LN-EDTA was not affected by soaking the substratum in 5 mM Ca²⁺ for 48 h before performing the centrifugal cell adhe-

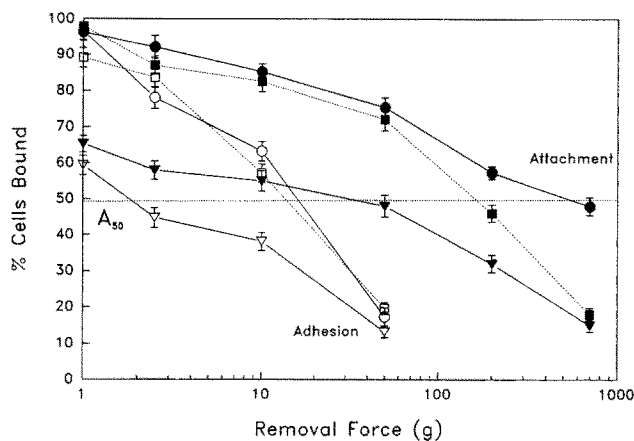


Figure 4. Comparison of neural crest cell attachment (37°C; closed symbols) and initial adhesion (4°C; open symbols) to LN-Ca²⁺ in the presence (circles) or absence (triangles) of divalent cations in the medium. The relative strengths of cell attachment and adhesion were measured by varying the removal force exerted on the cells adhering to laminin. Neural crest cell binding to laminin in the absence of divalent cations was normalized under the assumption that only 65% of the cells possessed these receptors (squares with dashed lines). Points represent the mean of at least six wells and the error bars represent the SEM.

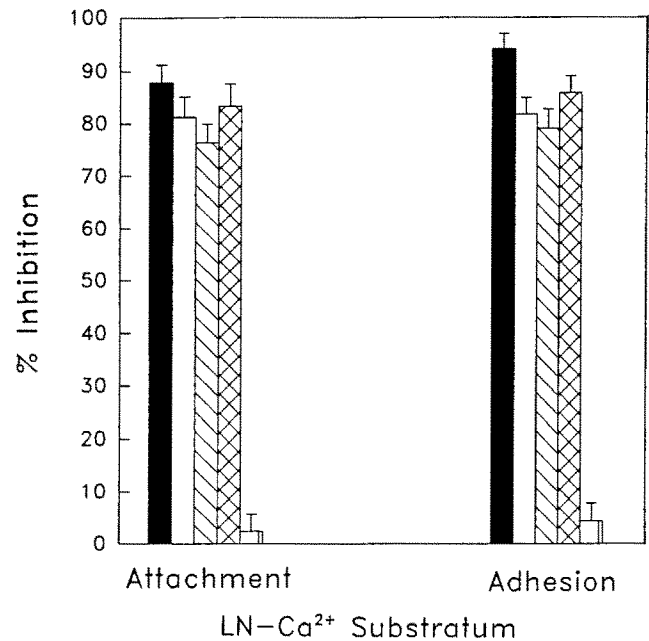


Figure 5. Effects of antibodies or heparin on neural crest cell attachment to LN-Ca²⁺ in the absence of soluble divalent cations. JG22 and HNK-1 antibodies were added to cells at 50 μg/ml, while heparin was added at 1 mg/ml. Anti-α₁ antisera was added at a dilution of 1:100. The removal force was 50 g. Bars represent the mean of at least six wells and the error bars represent the SEM. (■) JG22; (□) HNK-1; (▨) anti-α₁; (▩) anti-α₁ + HNK-1; (▨) Heparin.

sion assay, showing that this laminin substratum is stable once formed (data not shown).

Attachment and initial adhesion of neural crest cells to LN-Ca²⁺ were compared in the presence and absence of soluble divalent cations (Fig. 4). The attachment and adhesion of neural crest cells to LN-Ca²⁺ were significantly reduced by the removal of divalent cations from the media. The

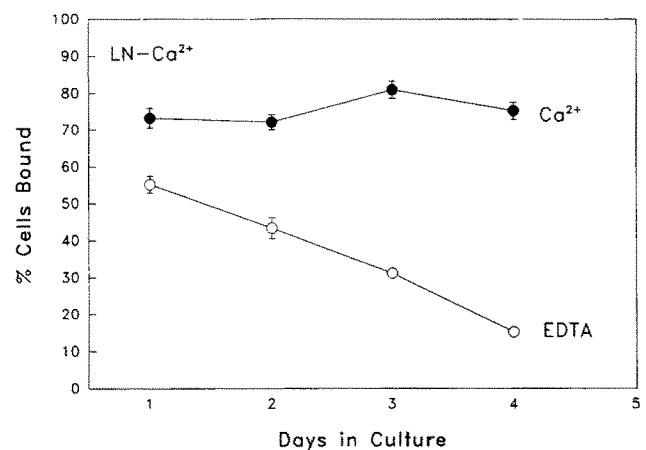


Figure 6. The effects of time in culture on the ability of neural crest cells to attach to LN-Ca²⁺ in the absence of soluble divalent cations. Neural crest cells were grown as primary cultures on fibronectin substrata for 1–4 d, removed from the plate, and assayed for their ability to attach to LN-Ca²⁺. Cell attachment was assayed in medium containing either Ca²⁺ or EDTA. The removal force was 50 g. Points represent the mean of at least six wells and the error bars represent the SEM.

avidity of attachment (A_{50}) was reduced from 500 g–50 g, while that of adhesion was reduced from 20 g–2 g. Even at minimal removal forces (1 g), the cation-independent attachment or adhesion to LN- Ca^{2+} never exceeded 65% of cells bound, in contrast to ~100% of cells bound in the presence of divalent cations. A possible explanation for this finding is that there may be two subpopulations of trunk neural crest cells, one possessing an integrin receptor for laminin that functions without soluble divalent cations (found in ~65% of the cells) and the other lacking it. When our data are normalized to consider 65% of the cells as one population (Fig. 5), the avidities for divalent cation-dependent and -independent adhesion are comparable on LN- Ca^{2+} .

The effects of antibodies and exogenous heparin in the medium were tested on attachment and initial adhesion to LN- Ca^{2+} (Fig. 5). JG22, anti- α_1 integrin and HNK-1 antibodies inhibited both of these processes in either the presence or absence of divalent cations; however, exogenous heparin had no significant effect. These results suggest that neural crest cells recognize LN- Ca^{2+} through a divalent cation-independent $\alpha_1\beta_1$ integrin. This integrin is completely inhibited by the HNK-1 antibody, suggesting that this receptor bears an HNK-1 epitope.

The attachment of trunk neural crest cells to LN- Ca^{2+} occurred in the absence of soluble cations when cells were cultured for 48 h or less. However, cation-independent attachment was subsequently lost with time in culture (Fig. 6). By 4 days in culture, trunk neural crest cell binding to LN- Ca^{2+} was entirely dependent on divalent cations.

Effects on Neural Crest Cell Attachment of Alterations in the LN- Ca^{2+} Coating Solution

Altering the Calcium Concentrations. To examine the effects of altering the Ca^{2+} concentration in the substratum coating buffer, laminin (2 or 20 $\mu\text{g}/\text{ml}$) was plated in the presence of varied concentrations of Ca^{2+} . For a given

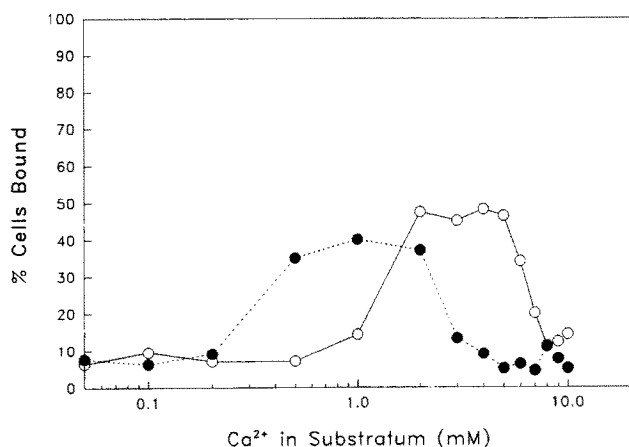


Figure 7. The effects of Ca^{2+} concentration in the laminin plating buffer on producing substrata that support divalent cation-independent attachment of neural crest cells. By varying the Ca^{2+} concentration for a set amount of laminin, the optimal Ca^{2+} concentration that supported divalent cation-independent attachment of neural crest cells was determined. Two concentrations of laminin were tested 20 $\mu\text{g}/\text{ml}$ (○) and 2 $\mu\text{g}/\text{ml}$ (●). The removal force exerted on the cells in this experiment was 50 g. Points represent the mean of at least six wells and the error bars represent the SEM.

laminin concentration, only a narrow range of Ca^{2+} concentrations within the coating buffer produced a substratum that could support neural crest cell attachment in the absence of soluble divalent cations (Fig. 7). At 20 $\mu\text{g}/\text{ml}$ of laminin, 2–6 mM Ca^{2+} produced a substratum that supported divalent cation-independent attachment of neural crest cells. At 2 $\mu\text{g}/\text{ml}$ of laminin, 0.5–2 mM Ca^{2+} produced an apparently similar substratum. These results suggest that the ratio of laminin to Ca^{2+} may be critical for creating a substratum conformation that supports cell attachment in the absence of soluble divalent cations.

Substitution of Other Divalent Cations. To test whether divalent cations other than Ca^{2+} can create substrata that support Ca^{2+} -independent adhesion and attachment, laminin was plated in the presence of 5 mM Mg^{2+} (LN- Mg^{2+}) or Mn^{2+} (LN- Mn^{2+}). In contrast to that observed on LN- Ca^{2+} , neural crest cell attachment to LN- Mg^{2+} , or LN- Mn^{2+} depended on the presence of Ca^{2+} (Fig. 8) or Mn^{2+} , but not Mg^{2+} (data not shown).

Addition of Glycosaminoglycans. We investigated the effects on cell attachment to laminin substrata of adding various glycosaminoglycans to the coating buffer. Coating solutions contained 20 $\mu\text{g}/\text{ml}$ laminin, 5 mM Ca^{2+} , and 500 $\mu\text{g}/\text{ml}$ of either heparin, dextran sulfate, or chondroitin sulfate. As shown in Fig. 9, inclusion of heparin in the coating solution removed the ability of the substratum to support divalent cation-independent neural crest cell attachment. In contrast, the addition of either dextran sulfate or chondroitin sulfate did not alter the ability of the substratum to support divalent cation-independent attachment. This suggests that the effects of heparin are not merely due to chelation of

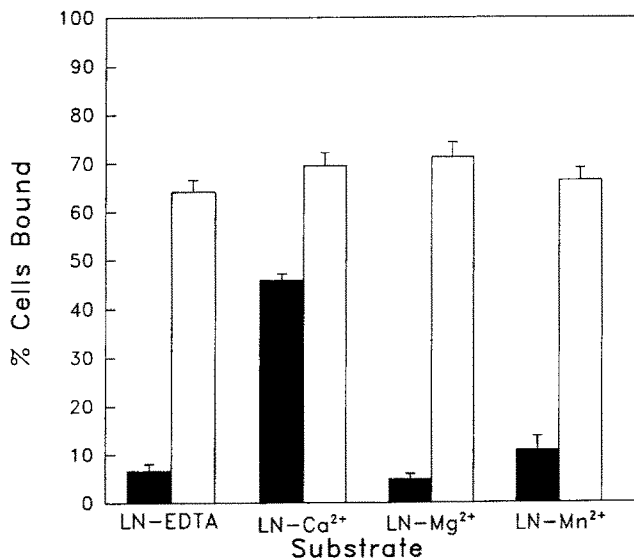


Figure 8. Neural crest cell attachment to various laminin substrata in the presence and absence of divalent cations within the medium. Laminin substrata were prepared by adding 1 mM of various divalent cations; Ca^{2+} (LN- Ca^{2+}), Mg^{2+} (LN- Mg^{2+}), or Mn^{2+} (LN- Mn^{2+}), or 1.5 mM EDTA (LN-EDTA) to substratum coating solutions containing 20 $\mu\text{g}/\text{ml}$ laminin. Either 1 mM Ca^{2+} (□) or 1.5 mM EDTA (■) was added to the cell attachment medium. The removal force exerted on the cells in this experiment was 50 g. Bars represent the mean of at least six wells and the error bars represent the SEM.

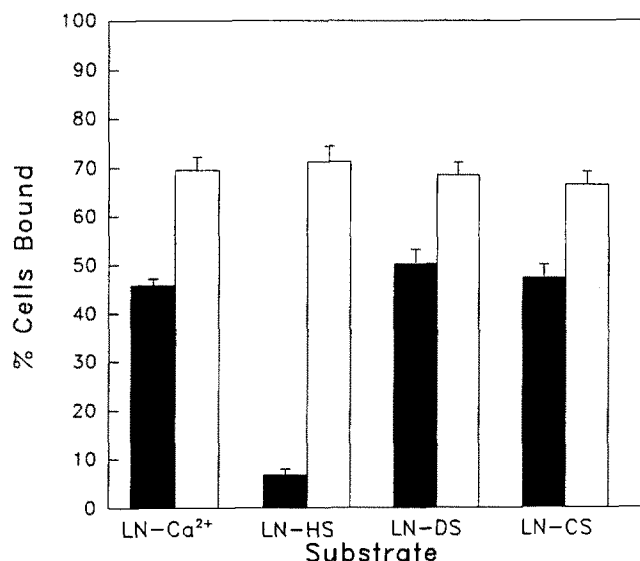


Figure 9. Neural crest cell attachment to various laminin substrata in the presence and absence of divalent cations within the medium. Laminin substrata were prepared by adding 1 mg/ml of heparin (LN-HS), dextran sulfate (LN-DS), or chondroitin sulfate (LN-CS) to substratum coating solutions containing 20 μ g/ml laminin and 5 mM Ca²⁺ (LN-Ca²⁺). 1 mM Ca²⁺ (\square) or 1.5 mM EDTA (\blacksquare) was added to the cell attachment medium. The removal force exerted on the cells in this experiment was 50 g. Bars represent the mean of at least six wells and the error bars represent the SEM.

Ca²⁺ ions by sulfate groups on the heparin. The heparin effect was concentration dependent. Low heparin concentrations (5 μ g/ml or less) did not abolish the divalent cation-independent attachment (~45% of cells bound; Fig. 10). At concentrations above 10 μ g/ml, attachment of neural crest cells was not significantly above background (~12%). In contrast, heparin within the coating solution had no effect on

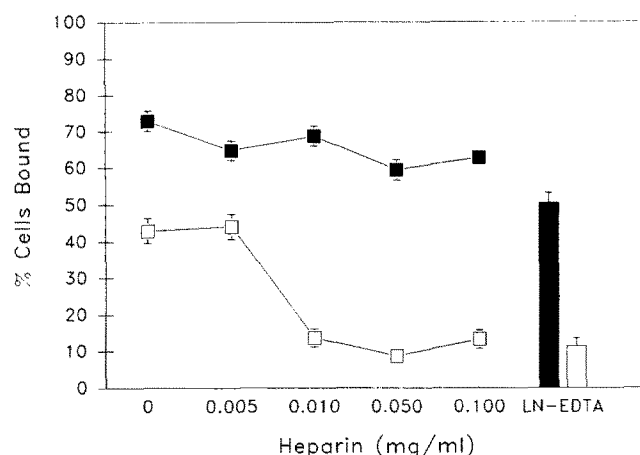


Figure 10. The effects of heparin on neural crest cell attachment to laminin in the presence or absence of divalent cations in the medium. Various concentrations of heparin were added to the laminin coating buffer containing Ca²⁺. Cell attachment was assayed in the presence of Ca²⁺ (\blacksquare) or EDTA (\square) within the medium. The removal force exerted on the cells in this experiment was 50 g. Points represent the mean of at least six wells and the error bars represent the SEM.

the divalent cation-dependent cell attachment to LN-EDTA ($p > 0.05$).

Neural Crest Cell Attachment on Alternating Lanes of LN-Ca²⁺ and LN-EDTA in the Presence and Absence of Divalent Cations

Adjacent lanes of LN-Ca²⁺ and LN-EDTA laminin substrata were prepared to compare directly the ability of neural crest cells to utilize these substrata. In the presence of divalent cations, neural crest cells showed no substratum preference, attaching and spreading equally well on LN-Ca²⁺ and LN-EDTA substrata (Fig. 11 A). However, 12 h after the medium was changed to remove divalent cations, neural crest cells were observed only on the LN-Ca²⁺ lanes (Fig. 11 B). Higher concentrations of EDTA in the medium resulted in sharper borders between the LN-Ca²⁺ lanes, which contained neural crest cells, and the LN-EDTA lanes, which were cell-free (Fig. 11 C). The number of neural crest cells per field on the LN-Ca²⁺ lanes increased with increasing concentrations of EDTA, whereas the number on LN-EDTA lanes decreased (Fig. 12). As the total cell number decreased from 100% to ~65%, there was a concomitant increase in the proportion of cells located on the LN-Ca²⁺ lanes, from 50% to nearly all of the attached cells. Thus, neural crest cells appeared to detach from LN-EDTA, but not LN-Ca²⁺ substrata in the presence of EDTA in the medium.

Identification of the 165 \times 10³ M_r Integrin on Neural Crest Cells

Surface biotinylated neural crest cells were immunoprecipitated using the HNK-1 antibody or the anti- α_1 antiserum and then transferred to nitrocellulose membranes for detection of bands using ¹²⁵I-streptavidin (Fig. 13 a). A major 165 \times 10³ M_r band was detected in both the HNK-1 and anti- α_1 immunoprecipitates. In addition, minor bands of 180 \times 10³ M_r and 120 \times 10³ M_r were observed. The chick α_1 subunit has been shown to have a M_r of 165 \times 10³ (Syfrig et al., 1991) while the chick β_1 subunit has a M_r of 120 \times 10³ (Horwitz et al., 1985). When the immunoprecipitates were again immunoprecipitated with the heterologous antibody (α_1 antiserum for the HNK-1 immunoprecipitates and HNK-1 antibody for the α_1 immunoprecipitates), identical bands were identified. When neural crest cells immunoprecipitated with HNK-1 or β_1 antibodies were subsequently immunoblotted with the α_1 antiserum (Fig. 13 b), two bands were observed at 165 \times 10³ M_r, corresponding to the α_1 subunit, and 120 \times 10³ M_r, corresponding to the β_1 subunit. By correlation with our functional data, these data suggest that the HNK-1 on neural crest cells is associated with the $\alpha_1\beta_1$ integrin heterodimer. Since the α_1 antiserum has some cross-reactivity with the β_1 subunit, we cannot distinguish unequivocally whether the epitope is on the α_1 and/or β_1 subunit.

Discussion

Our results demonstrate that neural crest cells attach to laminin by means of two receptor mechanisms that recognize distinct forms of laminin (Table I). Both mechanisms are medi-

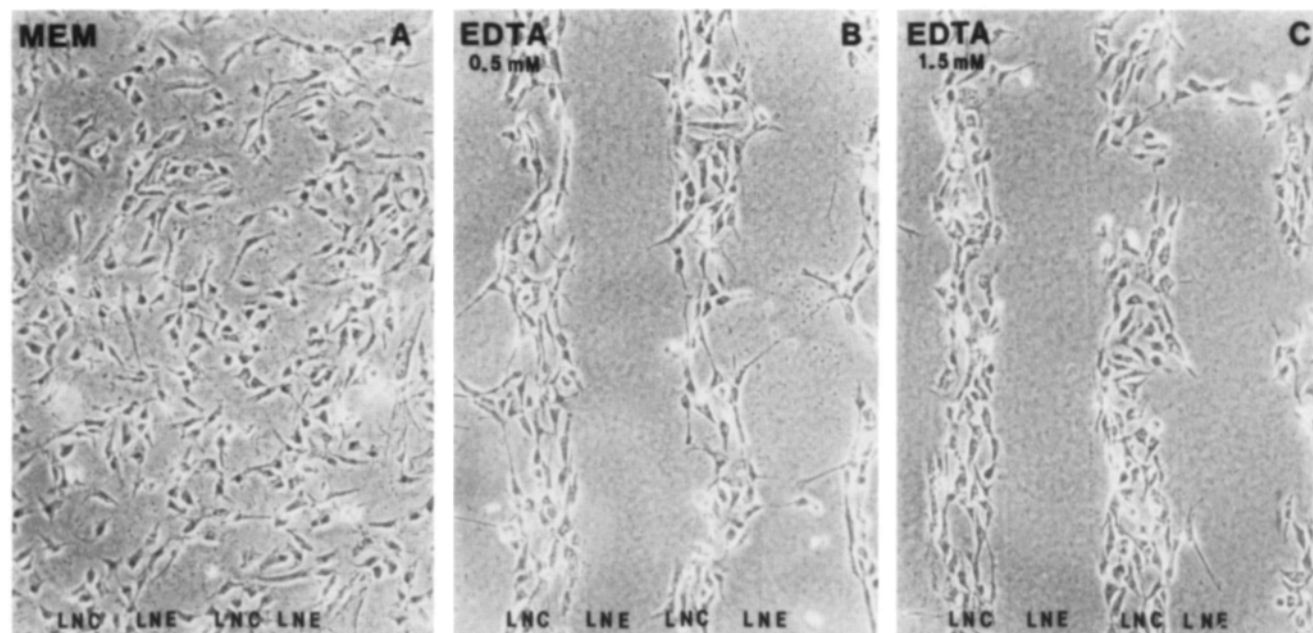


Figure 11. Comparison of neural crest cell attachment to adjacent lanes of LN- Ca^{2+} (LNC) and LN-EDTA (LNE) laminin substrata in medium containing divalent cations (A), 0.5 mM (B), or 1.5 mM (C) EDTA. (A) In the presence of soluble divalent cations, neural crest cells showed no substratum preference, attaching and spreading equally well on lanes of LN- Ca^{2+} and LN-EDTA substrata. (B) Twelve hours after addition of medium containing 0.5 mM EDTA, neural crest cells only were observed on the LN- Ca^{2+} lanes. (C) Addition of 1.5 mM EDTA resulted in a sharpening of the preference of neural crest cells for LN- Ca^{2+} over LN-EDTA lanes.

ated by integrin receptors, as evidenced by the complete elimination of neural crest cell attachment to all laminin substrata tested by antibodies to the avian β_1 subunit of integrin. One attachment mechanism is mediated by an $\alpha_1\beta_1$ integrin which functions in the absence of soluble divalent cations and has an associated HNK-1 epitope, as indicated

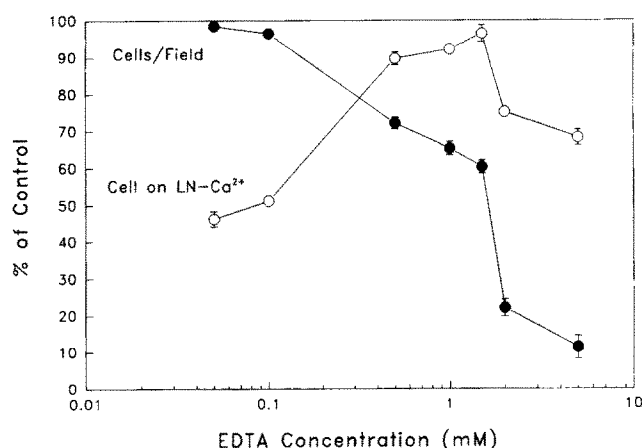
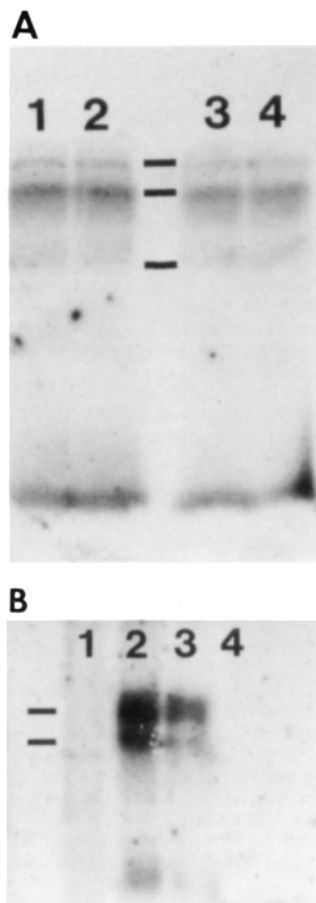


Figure 12. Number of neural crest cells per field on LN- Ca^{2+} and LN-EDTA substrata in the presence of increasing concentrations of EDTA in the medium. The number of cells per field on the LN- Ca^{2+} lanes increases with increasing concentrations of EDTA, whereas the number on LN-EDTA lanes decreases (○). The total number of cells per field is reduced to ~65% by adding 0.05–1.5 mM EDTA, and drastically drops above 2 mM EDTA (●).

by the finding that the anti- α_1 and HNK-1 antibodies block attachment of neural crest cells to LN- Ca^{2+} . Consistent with antibody-blocking experiments, the HNK-1 antibody recognizes 165×10^3 and $120 \times 10^3 M_r$ glycoproteins on neural crest cells isolated using either β_1 (Lallier and Bronner-Fraser, 1991) or α_1 integrin antibodies. The avian α_1 integrin subunit isolated from gizzard has been shown previously to have a molecular mass of 165×10^3 (Syfrig et al., 1991) and the β_1 subunit has an M_r of 120×10^3 (Horwitz et al., 1985). Although this is the first reported example of an integrin that functions in the absence of soluble divalent cations, our preliminary results suggest that a number of tissues possess divalent cation-independent integrins which mediate attachment to laminin, fibronectin, and collagens (our unpublished observations); this cation-independent attachment is lost as a function of developmental time. Because other investigators typically use adult cells or cell lines to examine the function of integrins, this class of integrins may have been missed in the past. Thus, studying a migratory embryonic cell type as opposed to a cell line yields important new insights into the mechanisms of integrin-mediated interactions. The second attachment mechanism to laminin is mediated by a different β_1 integrin, which requires either Ca^{2+} or Mn^{2+} , but not Mg^{2+} , for function. The α subunit of this heterodimer has yet to be identified.

The $\alpha_1\beta_1$ integrin heterodimer may be present on a subpopulation of neural crest cells. A maximum of 65% of neural crest cells were observed to attach to LN- Ca^{2+} in the absence of soluble divalent cations. This adhesion was inhibited by HNK-1 and/or α_1 antibodies whereas these anti-



itiate immunoblotted with the α_1 antiserum. Lane 3 is a JG22 immunoprecipitate immunoblotted with the α_1 antiserum. Lane 4 is a control in which the neural crest cells were omitted, but the sample was immunoprecipitated with JG22 antibody followed by an immunoblot with the α_1 antiserum. In lanes 2 and 3, the α_1 antiserum identified two bands of 165×10^3 (top line) and 120×10^3 M_r (bottom line), corresponding to the α_1 and β_1 subunits, respectively. This suggests that the HNK-1 epitope on neural crest cells is associated with $\alpha_1\beta_1$ integrin.

bodies only partially inhibited neural crest cell attachment to LN- Ca^{2+} in the presence of divalent cations. Taken together, these findings indicate that $\sim 35\%$ of trunk neural crest cells may lack the mechanism for divalent cation-

Table I. Summary of Results for Neural Crest Cell Interactions with Laminin

Substrata	Inhibitors				Divalent Cations			
	HNK-1	JG22	anti- α_1	Heparin	EDTA	Ca^{2+}	Mg^{2+}	Mn^{2+}
LN- Ca^{2+} (LN-CS) (LN-DS)	-	-	-	+	+	+	+	+
LN-EDTA (LN- Mg^{2+}) (LN- Mn^{2+})	+	-	+	+	-	+	-	+
LN-Heparin	+	-	+	+	-	+	-	+

+, Cell attachment; -, reduced cell attachment.

Figure 13. (A) Immunoprecipitates of neural crest cells using α_1 and HNK-1 antibodies. The HNK-1 antigen and the α_1 integrin of integrin were immunoprecipitated from surfaced-labeled neural crest cells. Immunoprecipitates using the HNK-1 antibody (lane 1) and anti- α_1 antiserum (lane 2) possess a major 165×10^3 M_r (middle line) band, and two minor 180×10^3 M_r (top line) and 120×10^3 M_r (lower line) bands. Subsequent immunoprecipitations of the HNK-1 immunoprecipitates with the anti- α_1 antibody (lane 3) or the α_1 immunoprecipitates with the HNK-1 antibody (lane 4) identify identical protein bands. **(B)** Immunoprecipitates of neural crest cells using β_1 and HNK-1 antibodies followed by immunoblots with the α_1 antiserum. Lane 1 is a control immunoprecipitate using no primary antibody, followed by an immunoblot with the α_1 antiserum. Lane 2 is an HNK-1 immunoprecipitate immunoblotted with the α_1 antiserum. Lane 3 is a JG22 immunoprecipitate immunoblotted with the α_1 antiserum. Lane 4 is a control in which the neural crest cells were omitted, but the sample was immunoprecipitated with JG22 antibody followed by an immunoblot with the α_1 antiserum. In lanes 2 and 3, the α_1 antiserum identified two bands of 165×10^3 (top line) and 120×10^3 M_r (bottom line), corresponding to the α_1 and β_1 subunits, respectively. This suggests that the HNK-1 epitope on neural crest cells is associated with $\alpha_1\beta_1$ integrin.

independent, HNK-1-sensitive attachment to laminin. Cell sorting experiments have shown that the HNK-1 antibody recognizes $\sim 60\%$ of trunk neural crest cells in culture (Maxwell et al., 1988), and these cells differ in their developmental potential from HNK-1⁻ neural crest cells (Maxwell and Forbes, 1991). It is tempting to speculate that there may be subpopulations of trunk neural crest cells that possess distinct ECM-receptor mechanisms, and later assume a different range of fates.

Previously, we showed that neural crest cells used different mechanisms of adhesion when the laminin concentration is altered in the presence of a constant amount of Ca^{2+} (Lallier and Bronner-Fraser, 1991). Here, we show that different adhesion mechanisms are invoked by altering the Ca^{2+} concentration for a constant amount of laminin. For a fixed concentration of laminin, only a narrow range of Ca^{2+} concentrations could support cation-independent attachment. Furthermore, LN- Mn^{2+} or LN- Mg^{2+} substrata cannot support divalent cation-independent attachment, suggesting a specific requirement for Ca^{2+} to obtain the LN- Ca^{2+} conformation. Thus, the ratio of laminin to Ca^{2+} in the coating buffer appears to be critical for producing substratum conformations that are differentially recognized by neural crest cells. Consistent with this, neurite outgrowth has been shown to be affected by the ratio of laminin to Ca^{2+} (Letourneau et al., 1988). Once formed, the various laminin substrata appear to be stable; this indicates that substratum conformation, rather than residual cations that may leach off from the substratum, dictate the mechanism of neural crest cell attachment.

Some insights into possible conformations of laminin have been gained by examining laminin self-assembly in solution and in planar substrata. Fluid turbidity and rotary shadowing experiments have shown that laminin self-assembly is temperature, Ca^{2+} , and heparin dependent (Yurchenco et al., 1985). When plated at 37°C in EDTA, laminin forms oligomers of three to five molecules which interact through their globular domains, while laminin plated in the presence of Ca^{2+} forms dense aggregates (Yurchenco et al., 1990). The binding of Ca^{2+} to laminin changes its sensitivity to proteolysis, again suggesting the occurrence of a conformational change. An analogous Ca^{2+} -dependent conformational change in the RGD-containing region of thrombospondin has been reported to affect the binding of $\alpha_v\beta_3$ integrin (Sun et al., 1992).

Heparin, but not other glycosaminoglycans, added to the substratum plating buffer can modify the laminin conformation in a dose-dependent manner, such that the resulting laminin substrata no longer support divalent cation-independent attachment even when plated in the presence of Ca^{2+} . These data indicate that heparin can act as a direct modulator of laminin conformation during the polymerization process. Because HSPGs are prevalent in the early embryo (Perris et al., 1991), co-localization of laminin and HSPG may influence substratum conformation under physiological conditions. In contrast, exogenous heparin has no effect on cell attachment once the substratum has been formed.

Our data together with known information on laminin polymerization (Yurchenco et al., 1985, 1990; Schittny and Yurchenco, 1990) suggest that laminin may exist in multiple conformations that are differentially recognized by neural crest cells. While it may be unlikely that changes occur in

the local Ca^{2+} concentration, these laminin configurations could be generated within the embryo by locally varying the relative concentrations of laminin, Ca^{2+} , and/or HSPGs. In vivo, neural crest cells encounter matrices that contain differential levels of laminin and HSPGs; for example, trunk neural crest cells migrate through the rostral half of each somitic sclerotome (Rickmann et al., 1985), a region rich in laminin (Krotoski et al., 1986; Duband and Thiery, 1987) but containing relatively low levels of HSPGs. In contrast, they generally do not penetrate basement membranes around the neural tube, dermomyotome, and under the epidermis (Weston, 1982; Erickson and Weston, 1983; Gehlsen and Hendricks, 1987; Erickson, 1988); these are rich in laminin and HSPGs (Krotoski et al., 1986; Lallier et al., 1990; Perris et al., 1991). Our data are consistent with the possibility that HSPGs may modulate recognition of laminin, since neural crest cells use distinct integrin receptors for LN- Ca^{2+} versus LN-Heparin. Although their avidity of attachment to the different laminin substrata is similar, neural crest cells can distinguish between substrata in the absence of soluble divalent cations. We cannot yet ascertain the functional relevance of these receptors for neural crest cell migration in vivo; however, it is possible that the cells could utilize these receptors to distinguish between different conformations of laminin. Because integrins can function in signal transduction (Dustin and Springer, 1989), it is possible that these receptor systems are involved in pathway selection between potential migratory substrata. Because integrins have numerous extracellular ligands, a number of molecules are likely to function as substrata for neural crest cell migration in vivo by a complex set of interactions. By analyzing neural crest cell-matrix interactions under defined and reproducible culture conditions, we can dissect the receptors present on neural crest cells and their ability to differentially recognize different ECM molecules.

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